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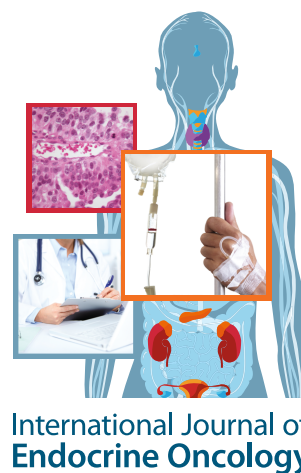
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Treatment-emergent neuroendocrine prostate cancer: molecularly driven clinical guidelines

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An increasingly recognized mechanism of prostate cancer resistance is the transdifferentiation from adenocarcinoma to treatment-emergent neuroendocrine prostate cancer (t-NEPC), an extremely aggressive malignancy. The incidence of t-NEPC has been increasing in recent years, in part due to novel treatments that target the androgen receptor pathway. While clinicians historically had very few options for t-NEPC detection and treatment, recent research has uncovered key diagnostic tools and therapeutic targets that can be translated into improved patient care. In this article, we will outline the clinical features of t-NEPC and its molecular pathogenesis. Importantly, we will also discuss recently uncovered molecularly based strategies aimed at improving the diagnosis and treatment of t-NEPC. Finally, we will propose a unified algorithm that integrates clinical and molecular information for the clinical management of t-NEPC.

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Overview of treatment-emergent neuroendocrine prostate cancer

Prostate cancer (PCa) represents the most frequently diagnosed malignancy in men. In a large proportion of cases, PCa can be detected prior to metastatic dissemination and, in such cases, treatments are initiated with curative intent. Unfortunately, about 10–20% of patients will develop recurrence following these therapies or will present with metastatic disease [1,2]. For these patients, the standard treatment is androgen-deprivation therapy, which elicits a significant response in the vast majority of cases. However, a large proportion of patients under androgen-deprivation therapy will develop castration-resistant prostate cancer (CRPC), a clinical entity that will require further antiandrogen therapy and/or taxane-based chemotherapy. Studies have demonstrated that CRPC cells can evade androgen receptor (AR) inhibition by several molecular mechanisms [3]. While some mechanisms are AR-dependent and can be therefore counteracted by second-line hormonal therapies (abiraterone and enzalutamide), increasing evidence suggests that hormonal resistance in the context of prolonged AR suppression can be mediated by transdifferentiation of adenocarcinoma cells into treatment-emergent neuroendocrine prostate cancer (t-NEPC).

While neuroendocrine PCa may arise *de novo*, the large majority of cases occur in patients with CRPC that have been treated with hormonal therapy and/or taxane-based chemotherapy [4]. Clinically, t-NEPC is an extremely aggressive malignancy that is resistant to current therapies used in the context of advanced prostate adenocarcinoma. In addition, t-NEPC displays high proliferative rates and tumor dissemination can occur quite rapidly [5]. Unlike CRPC, which tends to produce osseous metastases, t-NEPC typically disseminates to visceral organs such as lung and liver. Moreover, t-NEPC could be associated with distinct paraneoplastic syndromes, most notably hypercalcemia, Lambert-Eaton syndrome, Cushing's syndrome and syndrome of inappropriate antidiuretic hormone secretion [6]. However, it is worth noting that these syndromes are very rare and that no study has systematically

evaluated their differential incidence in NEPC versus prostate adenocarcinoma patients. Histologically, t-NEPC is usually characterized by small cells with prominent nucleus and limited cytoplasm that feature cytoplasmic eosinophilic granules, hyperchromatic nucleus, salt-and-pepper chromatin and a high proliferative rate. The immunohistochemical profile of t-NEPC includes the expression of neuroendocrine markers such as synaptophysin (SYP), chromogranin A (CHGA) and neuron-specific enolase (NSE), as well as absent AR and PSA expression [7,8].

Once considered a very rare occurrence, t-NEPC has become an increasingly recognized clinical problem. Recent evidence indicates that approximately one out of six patients with progressive hormone-resistant PCa has NEPC [3]. In keeping with this evidence, autopsy studies have shown that neuroendocrine *foci* may be present in about 10–20% of CRPC patients [6]. Given the extensive targeting of AR pathway and testosterone metabolism by recently developed drugs [9,10], the incidence of t-NEPC is expected to rise significantly in the near future. Unfortunately, t-NEPC is currently difficult to diagnose because it often arises in patients with multiple metastases, a condition that usually discourages clinicians from performing biopsies. In addition, current clinical guidelines lack a consensus definition of t-NEPC. As a result, the incidence of t-NEPC is usually underestimated and patients with undiagnosed t-NEPC are treated unnecessarily with the same regimen as patients with AR-positive prostate adenocarcinoma, with no success. Notably, preclinical and clinical evidence indicate that t-NEPC could be eminently sensitive to selected chemotherapies and targeted drugs. Hence, an appropriate classification of t-NEPC cases can have a transformative impact on patient treatment. Given the expected rise in t-NEPC incidence, it is imperative for clinicians to increase efforts to diagnose t-NEPC early and provide optimal oncological management.

In recent years, numerous studies have addressed the molecular profile of t-NEPC. Increasing evidence suggest that, while genetic mutations may contribute to its pathogenesis, t-NEPC is primarily driven by alterations that are epigenetic in nature. In addition, recent research on t-NEPC has also uncovered aberrant tyrosine kinase activity that can be therapeutically targeted. In this paper, we will critically discuss emerging evidence about the molecular pathophysiology of t-NEPC with an emphasis on clinically actionable alterations. Based on this information, we will also propose an integrated algorithm for the detection and management of t-NEPC in the context of advanced PCa.

Molecular pathogenesis

AR signaling

While there have been documented cases of small cell prostate carcinomas arising in noncastrated patients (*de novo* NEPC), the vast majority of patients present with t-NEPC following one or more cycles of hormonal therapy. Multiple lines of evidence support the model that t-NEPC cells are clonally derived from prostate adenocarcinoma cells. For instance, the frequency of TMPRSS-ERG fusion gene in CRPC closely resembles its frequency in NEPC, thereby pointing toward a common origin [11]. In addition, androgen-sensitive prostate adenocarcinoma cells have been shown to exhibit neuroendocrine differentiation in androgen-depleted cell medium [12], thereby implying that castration may actively promote the development of t-NEPC. Further suggesting a conserved underlying mechanism, reprogramming of normal prostate epithelial cells into neuroendocrine malignant cells has been described [13]. Interestingly, a similar lung epithelial to neuroendocrine transdifferentiation was also documented in the same manuscript [13]. Finally, xenograft experiments have shown that patient-derived xenografts from primary PCa can undergo neuroendocrine transdifferentiation following androgen deprivation [14]. In this model, androgen deprivation eliminates most but not all adenocarcinoma cells. The treatment-resistant adenocarcinoma cells enter a dormant state and after several weeks acquire NEPC features and the ability to proliferate in androgen-deprived conditions. Importantly, t-NEPC cells carry recurrent genetic and epigenetic alterations as an adaptive response, thus suggesting that key molecular pathways controlling cell fate may be used as targets for therapeutic intervention [14,15].

Genetic mutations

The advances in DNA sequencing have allowed an extensive genetic characterization of t-NEPC. Studies have shown that genetic inactivation of the retinoblastoma (*RB*), *p53* tumor suppressors and *PTEN* can be found in a majority of t-NEPC cases [16–18]. While *p53* and *PTEN* mutations are often present in CRPC, *RB1* loss appears to be more specific, since it was found in 90% of NEPC versus 15% of CRPC samples [16]. Other common genetic alterations in t-NEPC involve the amplification of *AURKA* and *MYCN* proto-oncogenes, which are concurrently present in more than 70% of metastatic t-NEPC cases versus 5% of unselected prostatic adenocarcinomas [19]. Notably, *AURKA* inhibitors have been developed and are currently in clinical trials for the treatment of NEPC [20,21]. Aside from

these mutations, it appears that there are few recurrent genetic alterations in t-NEPC. Since the neuroendocrine transdifferentiation is an active process that arises as a result of androgen deprivation, we believe that a mechanism that is not genetic but rather epigenetic is more probably the driving force in t-NEPC pathogenesis.

Epigenetic alterations

In addition to genetic mutations, epigenetic alterations have been well described in t-NEPC and appear to have a driving effect on neuroendocrine differentiation. Epigenetics refers to all heritable changes in gene expression that not attributed to changes in DNA sequence [22]. The drastic phenotypic transformation that drives t-NEPC is thought to be mediated by specific changes in transcriptional patterns, consistent with epigenetic plasticity. From a clinical standpoint, these driving epigenetic alterations may be used in the detection and pharmacological targeting of t-NEPC. In the following sections, we will discuss the main epigenetic alterations that have been shown to drive NEPC progression.

Polycomb group complexes

The most documented epigenetic alterations in t-NEPC have been the upregulation of the Polycomb group (PcG) protein EZH2. As the catalytic subunit of the Polycomb repressive complex 2 (PRC2), EZH2 trimethylates histone 3 at lysine 27 (H3K27me3) to induce gene silencing at target *loci* [23]. In t-NEPC, it has been shown that EZH2 is upregulated and can repress specific tumor suppressive genes, thereby favoring neuroendocrine transdifferentiation [24]. Furthermore, EZH2 has also been found to work in concert with N-Myc to promote tumor aggressiveness in t-NEPC [25]. Importantly, pharmacologic inhibition of EZH2 via the small molecule 3-deazaneplanocin A (DZNeP) has been shown inhibit the proliferation of AR-negative PCa *in vitro* and *in vivo* [26]. It should be noted that DZNeP has been subsequently identified as a broader histone methylase-inhibitor [27]. Hence, its effects on AR-negative PCa cells can be in part independent of EZH2 inhibition. More specific EZH2 inhibitors (e.g., tazemetostat) have shown promise for the treatment of refractory B-cell non-Hodgkin lymphoma and other advanced solid tumors [28], but are yet to be tested on NEPC models and patients.

Another PcG protein that has been implicated in t-NEPC emergence is the chromodomain protein CBX2. A member of the Polycomb repressive complex 1 (PRC1), CBX2 can directly bind to H3K27me3 via its chromodomain and further repress gene transcription at target *loci*, thereby acting as the bridge between PRC2 and PRC1 [29]. Furthermore, CBX2 can also mediate chromatin compaction in a PRC2-independent manner [30]. CBX2 is overexpressed at both the mRNA and the protein level in t-NEPC [15]. These results have been corroborated by a recent transcriptomic study comparing t-NEPC and CRPC clinical samples [3]. This study has shown that PRC2 components are among the top upregulated genes in t-NEPC.

In conclusion, it appears that aberrant PcG activity is a key feature of t-NEPC given the concurrent upregulation of CBX2 and EZH2 that also correlates with consistent downregulation of PcG target genes [15]. Interestingly, in lung cancer, CBX2 and EZH2 overexpression appears to be enriched in small cell carcinomas as opposed to non-small-cell lung cancer, further suggesting that overexpression of these epigenetic regulators promotes neuroendocrine-like phenotypes [15].

Heterochromatin proteins

Recently, a gene signature comprised of 36 heterochromatin-related genes has been shown to accurately distinguish NEPC from adenocarcinoma, thereby paving the way for accurate NEPC diagnosis [31]. Within this gene signature, HP1 α has been described as an early driver of NEPC transdifferentiation and proliferation [31]. HP1- α is a heterochromatic adaptor protein that can directly bind to histone H3K9me2/3 via its chromodomain and further recruit other heterochromatin-associated proteins via its 'chromoshadow' domain, thereby triggering chromatin compaction and gene silencing [32,33]. Interestingly, the epigenetic regulator DEK1, which controls heterochromatin integrity by interacting with HP1- α , has been identified as a driver of t-NEPC emergence [34,35]. Several of the 36 heterochromatin genes, such as HP1b, HP1r, SUV39H1 and DNMT1, are upregulated at later stages of t-NEPC transdifferentiation. All the aforementioned proteins have been described as HP1- α interactors [36–38]. These consecutive and concurrent events suggest that an HP1- α -dependent mechanism drives the coordinated activation of specific gene silencing programs in NEPC cells. Future investigations will need to identify the functional relevance of HP1 α -target genes in NEPC cells.

Noncoding RNAs

An important component of the cell's epigenetic machinery is constituted by noncoding RNAs (ncRNAs), in other words, transcripts that are not translated into proteins but regulate important cellular functions by interacting with specific macromolecules. Deep sequencing analyses have revealed that ncRNAs represent the vast majority of the human cell's transcriptome [39]. Importantly, ncRNAs orchestrate the coordinated activation of gene expression programs, thereby controlling cell differentiation, cell motility and other cancer-relevant phenotypes [40]. Given this background, it is not surprising that selected ncRNAs have been identified as drivers of t-NEPC transformation.

The most studied class of ncRNAs in this context is represented by miRNAs, short transcripts that bind specific mRNAs and inhibit their translation [41]. For example, an *in vitro* screening of miRNAs expressed during the transdifferentiation of prostate adenocarcinoma cells into NEPC cells has shown that this process is driven by the downregulation of *mir-17* and by the concurrent activation of its target, cyclin D1 [42]. A recent transcriptomic analysis of a murine NEPC model has identified two groups of microRNAs that drive different stages of the disease: when the cancer is localized to the prostate, a set of miRNAs activate Akt-dependent cell proliferation and survival; when the NEPC cells invade distant organs, a completely different miRNA signature is activated to suppress Akt activity and activate the epithelial-to-mesenchymal transition [43]. Notably, our studies on patient-derived xenografts confirmed the centrality of this pathway in advanced PCa; we have found that *mir-100*-dependent inhibition of mTOR (an indirect target of AKT) facilitates the postcastration survival of both CRPC and NEPC cells [44].

Another important component of the 'noncoding transcriptome' is represented by long ncRNAs (lncRNAs), which are defined as nonprotein-coding transcripts longer than 200bp. It is well established that these transcripts fold in tridimensional structures and interact with macromolecules in several ways, thereby controlling key cellular functions [40]. The role of lncRNAs in NEPC is still largely undefined but a few important players are emerging. A preliminary analysis of microarray and clinical data has identified the long ncRNA *MIAT* as an NEPC-specific transcript, whose expression is associated with poor prognosis [45]. Notably, *MIAT* is emerging as an oncogenic lncRNA in other neoplasms [46]. More recently, a large bioinformatic study has identified additional lncRNAs that are specifically expressed in NEPC cells. These include well-known oncogenic transcripts (e.g., *H19*) and several uncharacterized lncRNAs [47]. Further functional studies are needed to identify the mechanism of action of these NEPC-associated lncRNAs, as well as their translational relevance. Indeed, the expression of some lncRNAs is highly tissue- and disease-specific, making them ideal candidates for the development of noninvasive NEPC biomarkers [48].

Tumor microenvironment

While the genome and transcriptome of t-NEPC has been heavily investigated, recent evidence suggests that the tumor microenvironment may also harbor specific extracellular alterations that favor neuroendocrine differentiation and tumor dissemination. For instance, it appears that an acidic extracellular pH plays a role in t-NEPC cell survival as reduction in lactic acid secretion via MCT4 inhibition decreased t-NEPC cell viability [49]. The presence of stromal cells also plays a role in t-NEPC development, particularly for cancer-associated fibroblasts (CAFs). It has been demonstrated that heterogeneous populations of CAFs, characterized by alterations in SFRP1 and CD105, can directly induce neuroendocrine differentiation [50]. In keeping with this evidence, it was shown that epigenetic alterations in CAFs can induce a microenvironment that favors the neuroendocrine phenotype [51]. In addition, a prostaglandin-dependent inflammatory response has been shown to contribute to NEPC progression [52]. On the other hand, tumor infiltrating mast cells have been shown to inhibit NEPC transdifferentiation *in vivo*, possibly by reducing the local availability of stem cell factor [53].

Taken together, this evidence suggests that several adaptive molecular alterations occurring in the tumor microenvironment can trigger specific signal transduction pathways and epigenetic programs, thereby favoring t-NEPC initiation and progression. We have summarized this integrated molecular model of NEPC initiation in Figure 1.

Screening & diagnosis

Initial prostate biopsy

As previously discussed, NEPC may present *de novo* as a primary prostate malignancy, although this presentation remains very rare. In most cases, t-NEPC seems to rise from the transdifferentiation of prostate adenocarcinoma in patients exposed to prolonged hormonal therapy. However, as very few lesions are biopsied in these patients with advanced disease, t-NEPC is often underdiagnosed, no t-NEPC effective treatment is initiated and this causes

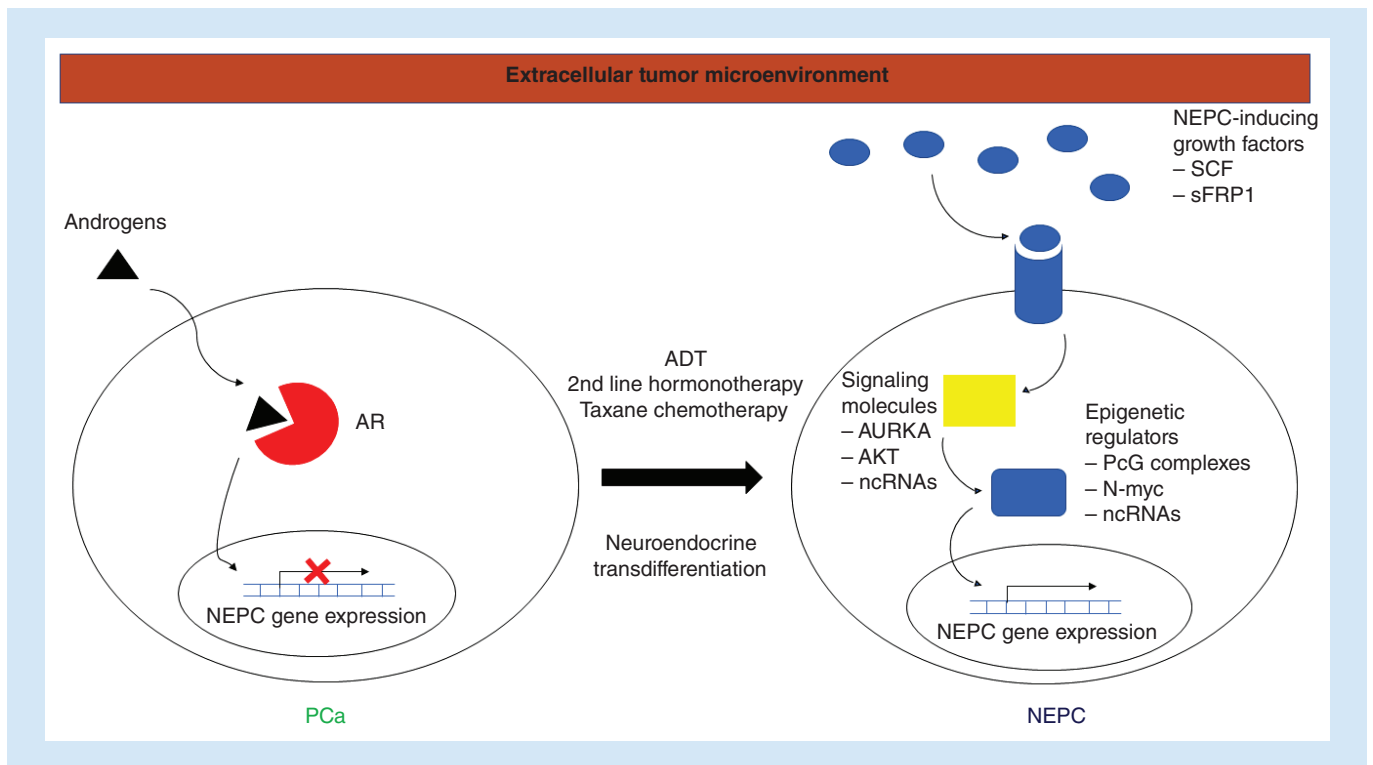


Figure 1. Molecular mechanisms involved in neuroendocrine prostate cancer pathogenesis. ADT: Androgen-deprivation therapy; AR: Androgen receptor; ncRNA: non-coding RNA; NEPC: Neuroendocrine prostate cancer; PCa: Prostate cancer.

rapid patient decline and death. Thus, it is imperative to identify and implement methods that can provide a fast and accurate diagnosis of t-NEPC.

It appears that some prostate adenocarcinomas are intrinsically predisposed to undergo neuroendocrine transdifferentiation because of their molecular profile. Studies in patient-derived xenograft models have shown that specific tumors can recurrently evolve to t-NEPC upon androgen deprivation, while others never transdifferentiate under the same conditions [14]. This implies that identifying the molecular alterations that predispose for neuroendocrine transdifferentiation could provide an estimated risk of developing t-NEPC. At present, no molecular alteration present in primary PCa is known to predict t-NEPC emergence upon androgen deprivation. However, further genomic and transcriptomic studies should explore this avenue since it would provide important information that allows earlier detection and treatment of t-NEPC.

Biopsy of metastatic tissues

Histological examination is currently the only method by which NEPC can be diagnosed. Almost all t-NEPC cases are characterized by extensive metastatic dissemination. Biopsies are rarely conducted in metastatic PCa cases because it is generally believed that the risks associated with this invasive procedure outweigh the possible benefits to the patient. However, we argue that biopsies should be performed more frequently and systematically in patients with advanced prostatic disease that present atypical progression for at least three reasons. First, t-NEPC generally carries a poorer prognosis than classical CRPC and does not respond to second-line hormonal therapies (abiraterone and enzalutamide). Since t-NEPC is intrinsically resistant to hormonal treatment, its early diagnosis can reduce the number of ineffective treatments targeting the AR axis, thereby reducing unnecessary costs and side effects. Second, the incidence of t-NEPC is increasing and is predicted to rise even more in the coming years [4]. Third, and most importantly, NEPC is more susceptible to chemotherapy regimens that are usually not employed in CRPC patients. In addition, some NEPC-targeted therapies are already in clinical trials and many more are investigated in preclinical models, thereby allowing unprecedented therapeutic options for this aggressive malignancy. For all these reasons, we strongly encourage clinicians to consider biopsy of progressing lesions that present atypical patterns.

An important notion to define is what to consider an atypical progression that warrants a biopsy. As previously stated, t-NEPC cells are AR-negative and do not express PSA. Thus, new metastatic nodules or rapidly progressing lesions without a proportional rise in PSA should prompt suspicion for t-NEPC. Another important difference between prostate adenocarcinoma and t-NEPC is the pattern of dissemination, as adenocarcinoma typically produces bone metastases while t-NEPC disseminates in visceral organs. Therefore, we recommend that visceral metastases in patients with CRPC should preferentially undergo biopsy of these metastases, especially if they occur without a proportional increase in PSA levels. Finally, another legitimate indication for biopsy is the presence of paraneoplastic syndromes associated with t-NEPC such as hypercalcemia, Lambert-Eaton syndrome, Cushing's syndrome and syndrome of inappropriate antidiuretic hormone secretion [6]. We believe all three of these clinical scenarios in a CRPC patient exposed to multiple lines of hormonal therapy should be considered 'red flags' for t-NEPC development and therefore warrant further investigation. As we will see in the following paragraphs, noninvasive diagnostic methodologies can be employed in the current clinical setting or in the near future to further stratify patients at risk of developing t-NEPC, thereby guiding the clinician's decision on when and if a biopsy should be performed.

Imaging

Imaging is a major technology for PCa detection [54]. The most commonly employed imaging methodologies are US, MRI and PET. Multiparametric US and MRI scans, in which different detection modalities are combined, have been shown to significantly improve the detection rate of PCas, when compared with conventional diagnostic strategies [55]. However, none of these technologies are currently able to distinguish prostatic adenocarcinomas from NEPCs. The use of artificial intelligence could improve the discriminatory power of these techniques in the future [56], thereby enabling the clinicians to perform noninvasive histologic analyses that would revolutionize the management of NEPC. However, until the technology is mature for these developments, MRI and ultrasound utility will be limited to performing guided biopsies and to identify visceral metastases, which are more frequently, but not exclusively, associated with NEPC.

Another interesting application of imaging techniques is the detection of metabolically active cancers using 2-deoxy-2-[fluorine-18]fluoro-D-glucose (FDG) PET scans, usually in combination with CT scans. These techniques proved to be an independent predictor of hormonal treatment failure in patients with metastatic CRPC. It would be intriguing to test the efficacy of this technique, possibly combined with other biomarkers, in detecting t-NEPC cases.

PET scans are less frequently used in the clinical setting, but they provide crucial information about the metabolic, cellular and molecular activity of cancer cells [57]. Of particular interest is the use of PET-based radiotracers that bind specifically PCa antigens. Clinical studies have successfully used radiolabeled antibodies or small molecule inhibitors of PSMA, a membrane protein overexpressed by PCa cells [58]. Since NEPCs are characterized by the specific expression of proteins such as CD56 and CHGA, it is conceivable that this technology will be adapted to the detection and localization of NEPCs in the coming years.

Taken together, this evidence indicates that multimodal US and MRI can already be used in the clinical setting to improve the accuracy of biopsies aimed at detecting t-NEPC. In the near future, we expect that targeted PET scans could represent a valuable, less invasive diagnostic tool.

Serum markers

The use of serum biomarkers, alone or in combination with imaging techniques, could provide an opportunity for noninvasive detection of t-NEPC as well as for monitoring of t-NEPC emergence in PCa patients treated with first- and second-line hormonal therapies. Four protein markers are currently used for the histological detection of NEPCs: CHGA, CD56, NSE and SYP [8]. All these markers can be detected by commonly used techniques (e.g., ELISA) in the serum of PCa patients. However, CHGA and NSE represent the most extensively studied serum biomarkers for NEPC detection and monitoring. Both CHGA and NSE seem to be progressively upregulated in serum samples of PCa patients exposed to hormonal therapies [59]. In addition, higher CHGA/NSE serum levels before abiraterone/enzalutamide treatment, or the elevation of these markers during the first 3 months of abiraterone treatment, are independent predictors of poorer clinical outcomes [60,61]. These results have been partially confirmed by a study showing that post-treatment elevation of serum CHGA levels predicts shorter progression-free survival (PFS) in CRPC patients treated with abiraterone [62]. Taken together, this evidence indicates that serum CHGA and/or NSE levels can be used to identify CRPC adenocarcinomas which transdifferentiate to NEPC during second-

line hormonal treatments. The evidence seems to be stronger for abiraterone and should be further confirmed in enzalutamide-treated patients.

Although these results are encouraging and in line with preclinical evidence on t-NEPC, the specificity and sensitivity of circulating NEPC protein biomarkers needs to be further tested. For example, a recent study showed that the serum expression of CHGA is unable to discriminate NEPC versus CRPC patients [3]. While serum NSE expression was significantly higher in NEPC versus CRPC patients, this marker showed a wide range of expression levels (5–90 ng/ml in the NEPC group vs 1–83 ng/ml in the CRPC group). This observation indicates that basal serum levels of NSE and CHGA cannot be used as reliable markers for t-NEPC detection. In addition, it should be noted that patients taking proton pump inhibitors have abnormally elevated circulating CHGA levels in the absence of neuroendocrine tumors. However, there is consistent evidence showing that an increase in CHGA and NSE predicts abiraterone resistance, and probably t-NEPC emergence in CRPC patients.

In addition to protein biomarkers, circulating nucleic acids can be used to improve the detection and monitoring of t-NEPC. As we have discussed, NEPC is characterized by a higher rate of mutations and gene copy number variations at specific loci. Hence, it is conceivable that these alterations can be detected in circulating tumor DNA (ctDNA). A recent study on enzalutamide- and abiraterone-resistant patients found that highly complex ctDNA signatures are associated with the emergence of treatment resistance [63]. So far, these findings have not been replicated by independent studies and it is not clear whether these complex signatures can be readily translated into the clinical setting. In addition, this study did not identify t-NEPC-specific ctDNA alterations. More promising results could come from the detection of NEPC-specific ncRNAs in serum samples, an approach that has been already used successfully to detect CRPC cases [64]. As our understanding of t-NEPC biology advances, we expect that more specific DNA and noncoding RNA biomarkers will emerge and be tested in clinical trials to improve the diagnostic and prognostic accuracy of serum NSE and CHGA.

Circulating tumor cells

Detection of neuroendocrine transformation of CRPC is currently dependent on tumor biopsy. Although tissue evaluation is the 'gold standard' diagnostic modality, access to tumor tissue is often very limited in this disease due to the nature of the metastases (often in visceral locations or bones), their location (pelvic or peri-vascular nodes), paucity and quality of tissue, in addition to morbidity from the biopsy procedure. Therefore, NEPC detection using minimally invasive blood-based markers would have high utility and allow longitudinal sampling in CRPC patients to provide early evidence of transdifferentiation to NEPC.

There is increasing evidence that circulating tumor cells (CTCs) in blood often indicate metastatic disease and have clinical potential as monitoring tools, prognostic markers, predictors of treatment efficacy including PFS and overall survival (OS). Their potential utility is very obvious as minimally invasive, real-time liquid bioassays with the possibility of representative characterization of early or advanced PCas at various stages and over time. From initial simple enumeration studies, there has been rapid technological progress to allow capture, enrichment and propagation of CTCs for downstream analyses including short-term cultures and *ex vivo* drug-sensitivity testing, single cell sorting of CTCs by fluorescence activated cell sorting, generation of xenografts, next generation sequencing, gene expression profiling, gene copy number determination and epigenomic analyses. Thus, CTCs can provide detailed phenotypic and molecular insights into progressive malignancies [64,65].

However, the key limitations of CTC based biomarker development include the extreme rarity of CTCs in blood (few as 1 in 10^9 blood cells), the variability in their size, shape and phenotypic markers. Technologies designed to isolate these cells [66] address the issues of recovery/isolation (enumeration), rate of throughput (volume of blood needed), purity (contamination with other cells) and clinical relevance (biological and therapeutic information obtained). The CTC isolation technologies currently available include antibody capture through expression of the cell adhesion molecule epithelial cell adhesion molecule, either through antibody-coated magnetic nanoparticles (CellSearch) or affinity based microfluidic devices (CTC Chip, Herringbone Chip, GO Chip). Other approaches avoid antibody-binding and allow for *in situ* staining or tumor cell recovery for analysis and include isolation by size of epithelia tumor through a filtration process, inertial based separation, Dean flow fractionation and dielectrophoresis [66].

Currently, the only test for CTC detection and enumeration with regulatory approval is based on immuno-magnetic enrichment of CTCs (CellSearch) expressing the epithelial cell adhesion molecule. CTC detection by CellSearch is prognostic for most men with metastatic PCa. CTC levels post-treatment with serial blood monitoring was associated with improved survival and reflected concurrent radiological responses and often predated falls in

PSA [67,68]. Two recent studies have addressed the potential utility of CTCs in NEPC. In both studies, NEPC transformation was confirmed by histology of a metastatic biopsy. The first, retrospectively, identified men with CRPC and available CTC enumeration (by CellSearch) and compared counts/7.5 ml blood with OS, measured from the first recorded CTC count until death or last follow-up. In 61 patients, (21 NEPC; 40 CRPC) the rate of detectable CTC counts was similar for the two groups (47.6% of NEPC and 55% of CRPC). The detection of 0–4 CTCs in NEPC was associated with a median OS of 22.6 versus 6.6 months in patients with ≥ 5 CTCs ($p = 0.001$). The authors concluded that CTC counts appeared to be prognostic for both NEPC and CRPC groups [69].

A further study used CellSearch and compared this with an alternative, nonselection bias platform (Epic Sciences; CA, USA) that characterizes all nucleated cells and identifies CTCs based on multiparametric digital reading of protein expression and cell morphology [70]. Epic identified distinct CTC populations and highlighted phenotypically heterogeneous CTCs from metastatic PCa patients. The study also used an additional molecular characterisation of these CTCs using FISH for AURKA, a gene commonly amplified in NEPC [71], showing concordance with matched metastatic biopsies. In selected cases CTCs were isolated from six patients using CellSearch and from all 17 NEPC patients with the Epic platform. NEPC-CTCs did express typical markers such as CHGA and SYP, but these were predominantly of smaller size compared with CRPC-CTCs and had lower AR expression as well as abnormal nuclear and cytoplasmic features. CRPC-CTCs also expressed lower levels of epithelial cytokeratins, possibly as a consequence of the epithelial-to-mesenchymal transition that can occur during metastasis and drug resistance [72,73]. These two studies have shown the enormous potential utility of CTC as serial monitoring tools in men at risk of NEPC following resistance and progression after AR-directed therapies for their CRPC. Further prospective studies should explore the clinical utility of routine CTC monitoring in the early and noninvasive detection of NEPC transdifferentiation.

Therapeutic avenues

Despite significant progress in defining and detecting NEPC, research has not yet led to improvements in treatment outcomes. To date, treatment for confirmed or suspected NEPC is a platinum-based regimen similar to those employed for the treatment of other neuroendocrine small cell carcinomas. These regimens have been studied in clinical trials for ‘variant’ and ‘anaplastic’ CRPCs, both of which are definitions that partially overlap with NEPC [71,72]. In these patients, response rates to cisplatin/carboplatin combinations with either docetaxel or etoposide are relatively high, but not durable. Addition of further conventional chemotherapeutics such as anthracyclines has not proven advantageous and has just increased toxicity [74]. In light of the increasingly recognized importance of t-NEPC, we believe that clinical trials specifically designed to identify the optimal composition and sequence of chemotherapy regimens in this cohort should be implemented as soon as possible.

In the more distant future, targeted therapies could substantially improve clinical outcomes in t-NEPC patients. Recent studies have shown that both the aurora kinase A (AURKA) protein is highly expressed and the *AURKA* gene amplified in NEPC [75]. Similarly, the N-myc proto-oncogene (*MYCN*) is both amplified and overexpressed in NEPC [19]. Subsequently, there is evidence that these proteins can cooperate and drive neuroendocrine transformation. Preclinical testing of an AURKA inhibitor, potentially also indirectly targeting N-myc, resulted in significant antitumor effects [75]. This has led to evaluation of alisertib, an AURKA inhibitor, in a Phase II clinical trial in patients with NEPC. Even though the study did not meet its primary end point of increased radiographic PFS, a subset of patients with activation of AURKA and N-Myc seemed to benefit from alisertib treatment [21]. We believe that these interesting results should be confirmed by a prospective study selectively enrolling NEPC patients with AURKA and N-Myc activation. The importance of N-Myc as a predictive marker is further highlighted by a recent study, which has identified a novel N-Myc–PARP–DNA damage response pathway in a subset of CRPC NEPC patients [76]. Using drugs already developed, this pathway may provide a further therapeutic opportunity in this disease [76].

As we have discussed, preclinical and clinical studies indicate that Polycomb genes are key drivers of t-NEPC initiation and progression. In this context, three EZH2 small molecule inhibitors (tazemetostat, GSK126 and CP-1205) are the most developed compounds. These three drugs have entered Phase I and II clinical trials for solid and hematologic malignancies [77]. Notably, a Phase I/II clinical trial is testing the combination of CP-1205 and abiraterone or enzalutamide in patients with metastatic CRPC (NCT03480646). In this context, it would be interesting to investigate whether CP1205 treatment suppresses the increase in CHGA/NSE serum levels, which is induced by second-line hormonal therapies [60], and if this phenomenon is accompanied by an enhanced efficacy of abiraterone and/or enzalutamide.

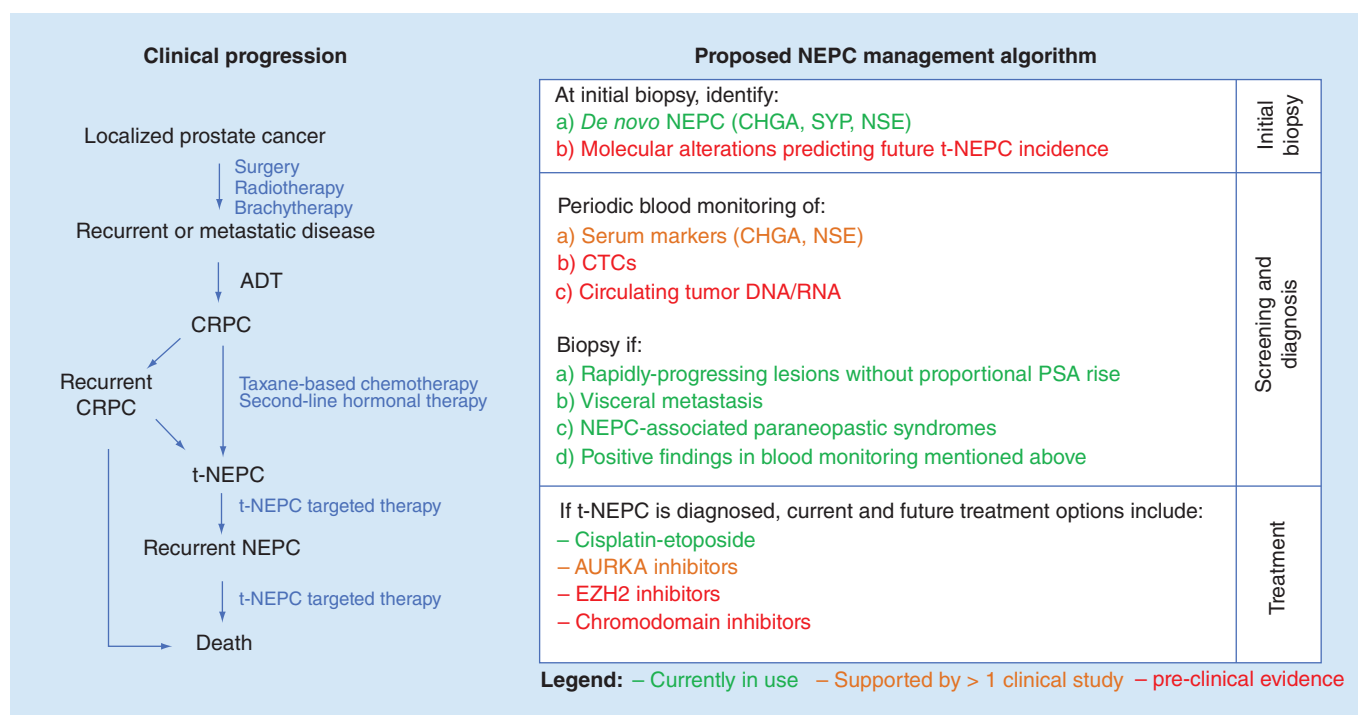


Figure 2. Clinical progression and proposed neuroendocrine prostate cancer management algorithm.
 ADT: Androgen-deprivation therapy; CTC: Circulating tumor cell; NEPC: Neuroendocrine prostate cancer.

Recently, there has been an interest in developing small molecule inhibitors of chromodomain-containing proteins such as the CBX proteins. Chromodomains bind to specific methylated lysine residues on histone proteins [78]. Therefore, by designing small molecules that can disrupt this interaction can interfere with the recruitment of aberrant epigenetic complexes such as PRC1 in the case of CBX proteins [79,80]. At this point, chromodomain inhibitors are only in preclinical testing, but some have demonstrated protein-specific activity with submicromolar affinity [81–84]. Given the involvement of CBX2 and HP1- α in t-NEPC pathogenesis [15,31], the development of chromodomain inhibitors may hold great promise for the treatment of neuroendocrine disease.

Taken together, this evidence suggests that in the near future clinicians will be able to treat t-NEPC patients with novel, more effective regimens, which could comprise both chemotherapy and targeted therapies. Within the NEPC cohort, patients could be stratified based on molecular markers and therefore assigned to a specific targeted treatment (e.g., EZH2 vs AURKA inhibitor).

A proposed algorithm of clinical management

Based on the literature discussed above, we propose a unified set of clinical guidelines for the management of NEPC (Figure 2). We believe that these guidelines are a work in progress, which will become more and more prescriptive with the identification of new biomarkers and effective therapies. For this reason, we have differentiated the clinical interventions based on three levels of evidence: interventions that are currently feasible in the clinical setting, interventions that have been proven useful in at least two clinical studies and interventions that are currently being investigated in preclinical models.

At initial biopsy, pathological examination of the prostate should always include the immunohistochemical markers of NEPC, such as CHGA, SYP and NSE. This allows the detection of *de novo* NEPC, which accounts for about 5% of prostate neoplasms and is resistant to localized PCa therapies and carries a much poorer prognosis [85]. Recent studies have shown that specific PCa types may be predisposed to undergo neuroendocrine transdifferentiation upon hormonal therapy [14]. Thus, mutations or gene signatures in primary PCa that can identify such patients represent important clinical tools that are currently under investigation.

Throughout the sequential progression of PCa, clinicians should always be on the lookout for t-NEPC and should consider biopsy whenever they suspect that t-NEPC may be present after evaluating the risks and benefits.

In particular, rapidly progressing lesions without a proportional PSA rise, in patients exposed to hormonal therapies, should prompt biopsy. Furthermore, biopsy should also be considered in CRPC patients with visceral metastases or NEPC-associated paraneoplastic syndromes such as those previously mentioned. Finally, clinicians should consider biopsy in the context of elevated serum markers such as CHGA and NSE. Although a baseline measurement of these markers does not carry high sensitivity [86], clinical studies indicate that an increase in circulating NEPC levels during hormonal therapy is more indicative of transdifferentiation and treatment resistance [60,61]. Hence, we believe that this hypothesis should be tested in prospective clinical trials and implemented in the clinical setting if confirmed. Currently, the analysis of CTCs or circulating tumor DNA/RNA has yet to become integrated in routine clinical practice, but we believe that these technologies hold great promise for the noninvasive monitoring of t-NEPC [70].

When t-NEPC has been confirmed, the first-line treatment should consist of platinum-based chemotherapy [6]. Unfortunately, this regimen does not lead to long-term remission and carries significant toxicity [6]. There are currently a number of drugs in development for t-NEPC but only Alisertib has been evaluated in clinical testing. We propose that specific pharmacogenetics tests should be designed in clinical trials evaluating the activity and efficacy of experimental targeted therapies. If these trials lead to positive results, platinum-based regimens could be rapidly associated with individualized targeted treatments. In conclusion, we believe that a combination of innovative diagnostic and therapeutic methods will lead to the rapid identification and effective treatment of t-NEPC, an underdiagnosed and lethal disease.

Conclusion

In conclusion, t-NEPC represents an extremely aggressive malignancy that occurs as a result of pharmacological targeting of the AR axis. With the recent introduction of novel hormonal treatments that potently suppress AR signaling and extend OS in CRPC patients, it is anticipated that the incidence of NEPC will rise in the near future. Thus, it is critical to provide clinicians with adequate tools to improve the clinical management of this lethal disease. Recent research has uncovered insights into the molecular pathophysiology of t-NEPC that may be translated into better diagnostic and therapeutic options. Although there remains some controversy within the definition of t-NEPC, this article provides an up-to-date summary of the literature addressing this emerging malignancy and provide the basis for its molecularly driven clinical management.

Future perspective

Emerging preclinical and clinical evidence corroborate the hypothesis that t-NEPC is a distinct clinical entity, which will require new diagnostic and therapeutic interventions. In this review, we have proposed a clinical algorithm to address this new clinical entity with currently available methodologies. However, we believe that, in the near future, the following challenges should be addressed by a concerted effort involving oncologists, pathologists and preclinical scientists:

- There is still no consensus definition of t-NEPC. Some pathologists call this type of PCa ‘anaplastic’ or ‘variant’. Hence, we think that a consensus conference involving leaders in the field from different disciplines should be organized to identify a shared working definition of t-NEPC
- Despite the presence of reliable tissue markers of t-NEPC, more work should be devoted to the identification of reliable circulating biomarkers. We believe that noncoding RNAs are particularly promising in this area
- Current treatment of t-NEPC is restricted to palliative chemotherapy. New agents (e.g., EZH2 inhibitors) should be tested in preclinical and subsequently clinical studies specifically designed for this type of malignancy

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Executive summary

- Overview: treatment-emergent neuroendocrine prostate cancer (t-NEPC) is an extremely lethal cancer that arises as a consequence of neuroendocrine transdifferentiation secondary to hormonal treatments in men with castration-resistant prostate cancer.
- Molecular pathogenesis: while very few genetic mutations can be recurrently found in t-NEPC, increasing evidence suggests that the pathophysiology of t-NEPC is epigenetic in nature and involves notably chromatin regulators and noncoding RNA.
- Screening and diagnosis: at present, diagnosis of t-NEPC is made on the basis of pathological findings of neuroendocrine differentiation in prostate tumors (local or metastatic) that have undergone hormonal treatment. Because this requires a biopsy and that few biopsies are performed in this clinical setting, t-NEPC is currently underdiagnosed. However, there are currently efforts to develop noninvasive methods of screening based on circulating tumor RNA/DNA, circulating tumor cells as well as imaging, notably using PET scans.
- Therapeutic avenues: classically, t-NEPC has been treated with platinum-based chemotherapy but unfortunately the oncological improvement is usually minimal with significant side effects. To date, the most advanced molecularly targeted therapy in clinical testing is alisertib (Phase II), an aurora kinase A kinase inhibitor. Other potential drug targets in investigation include MYCN, EZH2, CBX2 and HP1- α .
- Proposed clinical algorithm: integrating the latest clinical and preclinical data, we elaborate a set of clinical guidelines for the management of t-NEPC from its initial detection in patients with known prostate adenocarcinoma to its diagnosis and treatment.

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